MBHB Case No. 05-082

Generation of chemiluminescence by hydrogen

Cross Reference

[0001] This application claims the benefit of PCT application PCT/EP2003/009299 filed August 21, 2003 and German patent application, DE 102 39 098.3 filed August 26, 2002.

Background

[0002] Luminescent metal complexes are known from the prior art. EP-A-0 178 450 discloses ruthenium complexes that are coupled to an immunologically active material where the ruthenium complexes contain three identical or different or bicyclic or polycyclic ligands with at least two nitrogen-containing heterocycles, at least one of these ligands being substituted with at least one group such as SO_3H or -COOH which makes it water soluble and at least one of these ligands being directly substituted or substituted via a spacer group with at least one reactive group such as -COOH and the ligand being bound to the ruthenium by nitrogen atoms.

[0003] The use of luminescent metal complexes as labeling reagents for an electrochemiluminescence detection method is also known (cf. e.g. EP-A-0 580 979, WO 87/06706, US 5,238,108 or US 5,310,687). Such an electrochemiluminescence detection method is based on the conversion of the central atom of the metal complex e.g. ruthenium to the excited MLCT triplet state by electron transfer in a suitable measuring device. It can relax from this excited state by a forbidden triplet-singlet transition into the ground state with emission of a photon cf. e.g. WO/90 05296, Leland and Powell, J. Electrochem. Soc. 137 (1990), 3127-3131; Blackburn et al., Clin. Chem. 37 (1991), 1534-1539).

[0004] The reaction mechanism described in the literature for generating chemi-luminescence comprises the oxidation of a mediator such as tripropylamine to a radical cation. This radical cation loses a proton to become a TPA radical. The TPA radical is in turn the molecule which, by means of a further electron transition, converts an oxidized

metal complex e.g. a Ru^{3+} complex, into the Ru^{2+}-MLCT triplet state which is able to emit a photon.

[0005] However, the described mechanism cannot explain some experimental findings. Thus only 40-50 % of the theoretical current is found. Furthermore the generation of electrochemiluminescence is very dependent on the electrode material which, on the basis of the function of the electrode as an oxidizing agent of TPA and the metal complex, should not have been the case. In addition TPA dimers have also not been previously detected which should be formed in solution if TPA radicals are formed according to the above mechanism.

[0006] Hence further investigations were carried out on the generation of chemi-luminescence with metal complexes which surprisingly showed that a ruthenium complex in the presence of nascent hydrogen, e.g. generated by lithium/butanol/ H_2SO_4, exhibited chemiluminescence in a high yield.

[0007] On the basis of these new findings it is possible to provide a new method for generating chemiluminescence with a metal complex as the luminescence generator which comprises the use of nascent hydrogen to reduce oxidized metal complexes in the excited state that is capable of chemiluminescing. This method can be used especially to detect analytes in a sample thus improving the chemiluminescence yield or/and reducing the susceptibility to interference compared to previously used methods.

Summary of the Invention

[0008] The present invention concerns a method for generating chemiluminescence comprising the provision of a chemiluminescent species by nascent hydrogen. In particular the invention concerns a method for detecting an analyte in a sample using a luminescent metal complex as a labeling group and a device that is suitable therefor.

Detailed Description of the Invention

[0009] Hence a first aspect of the invention is a method for generating chemiluminescence with a luminescent metal complex as a luminescence generator

comprising the oxidation of the metal complex and reduction of the metal complex by nascent hydrogen to produce a form of the metal complex that can chemiluminesce.

[0010] In particular the invention concerns a method for detecting an analyte in a sample using a luminescent metal complex as a labeling group, wherein the luminescence of the metal complex is generated by the steps:

(i) oxidizing the metal complex;
(ii) reducing the metal complex by nascent hydrogen to produce a form of the metal complex that is capable of chemiluminescing; and
(iii) determining the analyte by means of the chemiluminescence.

[0011] Another aspect of the invention is a device for generating chemiluminescence using a luminescent metal complex as a luminescence generator comprising:

(i) means for oxidizing the metal complex and
(ii) means for generating nascent hydrogen.

[0012] In particular this device is intended to be used to detect an analyte in a sample using a luminescent metal complex as a labeling group comprising:

(i) means for oxidizing the metal complex,
(ii) means for generating nascent hydrogen and
(iii) means for detecting luminescence.

[0013] The method is particularly preferably used for applications in the field of diagnostics i.e. to detect an analyte in a sample. For example the method can be used to detect physical, chemical or biochemical parameters in a sample e.g. a body fluid, a tissue sample etc. or an environmental sample.

[0014] Detection of an analyte comprises contacting a sample with a detection reagent which carries a luminescent metal complex as a labeling group. The sample is preferably a biological sample and is present in a liquid form. It can be derived from human, animal or plant tissues, body fluids, prokaryotic or eukaryotic cell cultures etc.

[0015] The detection reagent comprises a luminescent metal complex as a labeling group which is preferably coupled to a biological substance e.g. biotin, nucleic acids, e.g. oligonucleotides, DNA or RNA, nucleic acid analogues such as peptidic nucleic acids, antibodies or antibody fragments, peptide or polypeptide antigens i.e. immunologically reactive polypeptides or haptens i.e. organic molecules having a molecular weight of 150 to 2000, and optionally other reagents that are known to a person skilled in the art.

[0016] The procedure for the detection method according to the invention preferably comprises an incubation of the sample with the detection reagent in order to directly or indirectly react the detection reagent with analytes present in the sample. The presence or amount of an analyte in the sample is determined qualitatively or/and quantitatively on the basis of the chemiluminescence signal originating from the labeling group.

[0017] The method can be carried out as a homogeneous assay i.e. the chemiluminescence is measured in a liquid phase. However, it is preferable to carry out a heterogeneous test in which the chemiluminescent label is immobilized on a solid phase e.g. a particulate solid phase such as magnetic microbeads e.g. streptavidin-coated microbeads or on colloidal particles. When carrying out a heterogeneous test, the method according to the invention can include so-called capture and washing steps in which the label is immobilized on the solid phase and the other sample components are separated.

[0018] A preferred feature of the method according to the invention is the use of chemiluminescent metal complexes which contain a structure of the general formula (I):

$$[M(L_1L_2L_3)]_n\text{-}Y_m\text{-} \qquad (I)$$

[0019] in which M is a divalent or trivalent metal cation selected from rare earth or transition metal cations,

L_1, L_2 and L_3 are the same or different and denote ligands containing at least two nitrogen-containing heterocycles, L_1, L_2 and L_3 being bound to the metal cation by nitrogen atoms,

[0020] Y denotes a linker bound to one of the ligands by means of which the complex (a) is coupled to a biological substance or (b) can be coupled to a biological substance, m is an integer from 1 to 10, preferably from 1 to 4 and is particularly preferably 1 and n is an integer from 1 to 6, preferably from 1 to 3 and is particularly preferably 1.

[0021] The metal cation in this complex is preferably ruthenium, osmium, rhenium, iridium, rhodium, platinum, indium, palladium, molybdenum, technetium, copper, chromium, tungsten, yttrium or lutetium. Ruthenium, iridium, rhenium, chromium and osmium are particularly preferred. Ruthenium is most preferred. The complex can optionally additionally contain counterions e.g. anions for charge equalization.

[0022] The ligands L_1, L_2 and L_3 are preferably ligands containing at least two nitrogen-containing heterocycles. Aromatic heterocycles such as bipyridyl, bipyrazyl, terpyridyl and phenanthronyl are preferred. The ligands are particularly preferably selected from bipyridine and phenanthroline ring systems.

[0023] Hydrophilic groups or/and charge carriers which are for example covalently bound e.g. to the linker or to another substituent of the ligands L_1, L_2 or L_3 are particularly preferably present in the metal complexes according to the invention. Such hydrophilic or charged metal complexes are known for example from WO 96/03409 and WO 06/03410. In the sense of the present invention the term "charge carrier" means a group which is present predominantly in an ionic form at a pH in a range of 6 to 8. The complex preferably contains up to 10, particularly preferably 2 to 8 such charge carriers.

[0024] The complex particularly preferably contains at least one negative charge carrier. Examples of suitable negative charge carriers are phosphate, phosphonate, sulfonate and carboxylate groups, where sulfonate and carboxylate groups are most preferred.

[0025] Complexes which contain a hydrophilic group are also suitable for the method according to the invention. Examples of suitable hydrophilic groups are C_2-C_3 alkyleneoxy units, C_2-C_3 alkylenethio units and polyhydroxy units.

[0026] Such metal complexes can be produced by known methods, for example by reacting a metal salt e.g. a metal halogenide and optionally subsequently exchanging the halogenide ion for hexafluorophosphate, trifluoroacetate or tetrafluoroborate groups. Such methods are known. The metal complex is usually used for the method according to the invention in the form of conjugates with a biological substance in which at least one metal complex is coupled to the biological substance. Examples of suitable biological substances are cells, viruses, subcellular particles, proteins, lipoproteins, glycoproteins, peptides, polypeptides, nucleic acids, oligosaccharides, polysaccharides, lipopolysaccharides,

cellular metabolites, haptens, hormones, pharmacological agents, alkaloids, steroids, vitamins, amino acids and sugars.

[0027] The metal complex is preferably coupled to the biological substance by means of a reactive or activatable functional group on the metal complex e.g. a carboxylic acid halogenide, a carboxylic acid anhydride or an active ester such as an N-hydroxy-succinimide ester or a maleimide which can covalently couple to a functional group of the biological substance. If the functional group is a carboxylic acid anhydride, carboxylic acid halogenide or active ester, it can for example be coupled to free amino groups of the biological substance. If the functional group is a maleimide residue, it can couple to free SH groups of the biological substance. Functional groups of the biological substance can also be activated in a similar manner and these functional groups can subsequently for example react with a free carboxylic acid, amino or thiol group of the metal complex.

[0028] The method according to the invention comprises the steps (i) oxidizing the metal complex and (ii) reducing the metal complex. The oxidation of the central atom of the metal complex can take place electrochemically or chemically. For the electrochemical oxidation an adequate anodic potential for the respective metal ion is applied to an electrode. For the transition Ru^{2+}/Ru^{3+} this potential is preferably at least +1.2 V, particularly preferably + 1.2 to + 1.4 V (relative to an Ag/AgCl reference electrode). Alternatively the central atom of the metal complex can also be chemically oxidized. Examples of suitable chemical oxidizing agents are PbO_2, permanganate, Cer^{4+} compounds or/and peroxodisulfates.

[0029] In the case of a prior chemical oxidation the subsequent reduction is preferably spatially separated or/and separated in time and can for example take place in two separate reaction chambers where the oxidation is carried out in the first reaction chamber and the reduction is carried out in the second chamber. Excess oxidizing agent is preferably removed before the reduction e.g. by removal or/and – in the case of a heterogeneous test with a solid phase-bound labeling group – by washing the solid phase. Alternatively an excess of the oxidizing agent can also be destroyed by a third substance.

[0030] If an electrochemical oxidation of the metal complex is carried out, the method can be carried out in a single chamber in which nascent hydrogen is generated or/and introduced during the reduction step.

[0031] The reduction step in the method according to the invention comprises the generation of nascent hydrogen in order to convert the oxidized metal complex into a state that allows the emission of a chemiluminescence photon. In order to maximize the efficiency of the reduction, it is preferred that the nascent hydrogen is formed in the direct vicinity of the metal complex and in particular at a distance of no more than 50 nm. The nascent hydrogen can be generated electrochemically, chemically or/and by ultrasound. Electrochemical generation of the nascent hydrogen is preferably carried out by applying a voltage of $\leq$-1.0 V (relative to an Ag/AgCl reference electrode). Nascent hydrogen can be chemically generated using known reagents such as Li/butanol/H_2SO_4, Zn-Cu/ethanol or Zn/HCl. Generation of nascent hydrogen by means of ultrasound is preferably carried out by detaching or expulsing hydrogen radicals from organic compounds and in particular from alkyl compounds. In this case the ultrasonic energy is preferably in the range of 0.1 – 10 MHz, particularly preferably about 1 MHz (Suslick & Price, Annu. Rev. Mater. Sci 29 (1999), 295; Mizik & Ries, Ann. NY Acad. Sci 899 (2000), 335).

[0032] A particularly preferred embodiment of the method according to the invention comprises firstly a chemical oxidation of the metal complex and subsequently an electrochemical generation of nascent hydrogen, e.g. in an electrochemical cell, which provides the nascent hydrogen at a high concentration. Examples of suitable electrochemical cells are described in EP-A-0 658 760. Also in this embodiment it is expedient that the oxidation and generation of nascent hydrogen take place in two separate reaction chambers.

[0033] The present invention is further elucidated by the following example:

Example Chemiluminescence by nascent hydrogen

[0034] A ruthenium (bipyridyl)$_3$ complex (containing a Ru^{2+} cation) was oxidized to a Ru^{3+} complex. A homogeneous system comprising Li/butanol/H_2SO_4 was used for this. Concentrated sulfuric acid was added to a vessel of PbO_2 (powder) and it was overlayered with butanol. The Ru^{2+} complex was dissolved in the butanol at a concentration of 1 mmol. The Ru^{2+} complex is oxidized at the interface between H_2SO_4 and butanol. After the

oxidation of the Ru^{2+} complex to Ru^{3+} had taken place, lithium was added. In this process nascent hydrogen is formed at the interface between H_2SO_4 and butanol. A pronounced ruthenium chemiluminescence was observed at this interface.

[0035] Ru chemiluminescence was also observed when Ru^{2+} was electrochemically oxidized to Ru^{3+} and nascent hydrogen subsequently generated by Li/butanol/H_2SO_4.